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p53 Protein Immunoexpression in Esophageal Squamous Cell Carcinoma and Adjacent Epithelium

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Background: Immunoreactivity for p53 tumor supressor gene product is commonly found in human malignancies and some premalignant lesions, but its role in cancer development and its value as a marker of tumor biologic behavior is still unclear.

Objectives: This study was undertaken to assess p53 immunoexpression in esophageal squamous cell carcinoma and attempts to determine its correlation with morphological features associated with tumor behavior.

Methods: Immunohistochemical study was performed on archival paraffin-embedded tissue of 37 esophageal squamous cell carcinomas and respective adjacent mucosa.

Results: Twenty-one tumors (56.8%) demonstrated specific staining for p53. Sixteen areas of dysplasia were present in 14 out of the 35 cases. p53 positivity was found in one low-grade dysplasia and in six high-grade dysplasias. By univariate analysis, p53 immunoexpression correlated positively with local invasion ($P = 0.01$) and perineural spread ($P = 0.04$). Multivariate analysis with logistic regression showed that tumor invasion was the only factor that discriminated between p53 positive and p53 negative cases (OR:15.6, $P < 0.02$). No relationship was found between p53 expression and tumor grade, DNA nuclear ploidy, and S-phase fraction.

Conclusions: These data suggest that p53 dysfunction may be implicated in early, preinvasive, stages of esophageal cancer as well as in the tumor progression related to a more invasive phenotype.

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KEY WORDS: **p53; esophageal neoplasms; squamous cell carcinoma; dysplasia; prognosis**

INTRODUCTION

p53 is a tumor supressor gene located in the short arm of the chromosome 17, whose wild-type product prevents uncontrolled cellular proliferation after DNA damage through a G1 arrest checkpoint [1].

Alterations in the p53 structure represent one of the most common genetic changes associated with human cancer [2]. The wild-type p53 gene product can be inactivated by mutation, allelic deletion, as well as by complex formation with mutant p53, virus, and aberrant host-binding proteins [3–5]. The short half-life of the wild-type p53 product does not allow its immunohistochemical detection, but mutant p53 protein usually has a

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higher stability and prolonged half-life, being identifiable by immunohistochemistry techniques. p53 immunoexpression is associated with loss of normal protein function, and it was demonstrated in tumors and cell lines to correspond to a mutated form of the gene product [6]. However, there are p53 mutations associated with non-stable protein that remain undetected if immunohistochemistry is the only evaluation method used [2].

Loss of normal p53 function seems to be a critical molecular event in human tumorigenesis, but there is a considerable discrepancy in the time or stage of oncogenesis at which p53 gene mutation have its effect [7]. Likewise, there are conflicting results on the utility of the assessment of p53 mutation and tissue expression for the purpose of tumor prognosis [8–16]. p 53 immunoexpression has been described in premalignant and hyperplastic squamous cell lesions of the larynx [17], esophagus [18–20], and uterine cervix [21], but the role of altered gene protein in the pathobiological mechanisms of malignant transformation remains unclear.

This study was undertaken to identify p53 protein accumulation in squamous cell carcinoma of the esophagus and corresponding adjacent mucosa, attempting to ascertain the role of p53 in the process of malignant transformation. Additionally, we evaluated the association of p53 alterations, as assessed by immunocytochemistry, with epidemiological features implicated in esophageal carcinogenesis as well as with histological parameters of prognostic value.

TABLE I. Clinicoepidemiological Features of the p53 Positive and Negative Groups of Patients With Esophageal Squamous Cell Carcinomas

	p53 positive (%)	p53 negative (%)	*P* value
Sex			NS
Male	20(95.2)	11(68.8)	
Female	1(4.8)	5(31.2)	
Mean age	62.1 ± 10.7	59.8 ± 10.6	NS
Location[a]			NS
UO	1(4.8)	1(6.3)	
MTO	10(47.6)	10(62.5)	
LTO	10(47.6)	5(31.2)	
Stenosis			NS
yes	8(61.5)	6(42.9)	
no	5(38.5)	8(57.1)	
Alcohol			NS
<40 grams/day	1(8.4)	0(0.0)	
40–80 grams/day	4(33.3)	1(16.6)	
80–160 grams/day	3(25.0)	4(66.7)	
>160 grams/day	4(33.3)	1(16.7)	
Tobacco			0.02
<10 cigarettes/day	4(28.6)	0(0.0)	
10–20 cigarettes/day	1(7.1)	4(66.7)	
20–40 cigarettes/day	5(35.7)	2(33.3)	
>40 cigarettes/day	4(28.6)	0(0.0)	
Radiotherapy			NS
yes	8(38.1)	3(18.8)	
no	13(61.9)	13(81.2)	

[a]UO-cervical and upper thoracic esophagus; MTO-medium thoracic esophagus; LTO-lower thoracic esophagus.
NS-not significant.

MATERIALS AND METHODS

Patients and Clinical Data

Thirty-seven patients submitted to esophagectomy because of squamous cell carcinoma, at the Instituto Português de Oncologia-Lisbon Center, between 1986 and 1990, were included in this study. Clinicoepidemiological data (Table I) were obtained from the medical records. The mean age of the patients, 31 males and 6 females, was 61 years (range: 39–78 years). The locations of the tumors were as follows: upper thoracic including cervical (n = 2, 5.4%), middle thoracic (n = 20, 54.0%) and lower thoracic (n = 15, 40.5%). Twenty-seven patients had stenosis, assessed by endoscopy; alcohol and tobacco consumption were evaluated in 18 and 20 cases, respectively, using a four grade scoring; 11 patients received pre-operative radiotherapy (Table I).

Histopathological Study

Formalin-fixed, paraffin-embedded tissue from the tumor areas and adjacent mucosa was used for the assessment of p53 immunostaining and cytometric analysis. All the samples were selected on hematoxylin-eosin stained slides and included representative areas of neoplastic tissue and esophageal mucosa with and without dysplastic changes. The tumors were classified and staged according to the World Health Organization [22] and the American Joint Cancer Committee [23] criteria, respectively.

The neoplasia were graded as follows: well differentiated (27; 73.0%), moderately differentiated (7; 18.9%) and poorly differentiated (3; 8.1%). Eight cases had no invasion behind the tunica muscular propria: one neoplasm (2.7%) was limited to the submucosa (T1) and seven (18.9%) showed invasion of the muscular propria limited to the external layer (T2). In 29 cases, there was invasion below the muscular propria: in 27 cases (73.0%) it involved the adventitia (T3) and in two cases (5.4%) the tumor infiltrated adjacent structures (T4). Seventeen tumors had no regional lymph nodes metastasis (NO; 45.9%) and an equal number of cases had positive nodes (N1; 45.9%). In three cases the information available was insufficient to assess the lymph nodes status (NX). None had distant metastasis (MO) at the time of operation. Venous invasion, lymphatic permeation, and perineural spread were assessed in all the cases. Twenty-seven tumors (73.0%) showed permeation of the lymphatic vessels, four (10.8%) had venous invasion, and there was perineural tumor spread in 17 (45.9%).

Adjacent mucosa was studied in all but two cases, for cytopathic viral changes. Twelve of 35 cases showed koylocytic changes suggestive of human papilloma virus (HPV) infection.

Fourteen of 35 cases presented dysplastic changes adjacent to carcinoma in a total of 16 foci. They corresponded to foci of low-grade dysplasia, present in five areas, and to atypical epithelium with features of high grade dysplasia in 11 areas. Two cases had concomitant low-grade and high-grade areas.

Immunocytochemical Study

For antigen retrieval, the immunohistochemical study was performed using a microwave at 500 W, during 15 minutes [24]. Five-micron-thick, formalin-fixed, paraffin-embedded sections were dewaxed and endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Sections were incubated with a mouse monoclonal antibody anti-p53 product (DO7-DAKO, Glostrup, Denmark), at a 1:400 dilution. The sections were washed in Tris buffered saline (TBS), incubated for 30 minutes with biotinylated rabbit antimouse immunoglobulin G (IgG) (DAKO-Glostrup, Denmark) at a 1:250 dilution and incubated with streptavidin-biotin peroxidase complex (DAKO-Glostrup, Denmark) for 30 minutes. The enzyme was visualized using diamino benzidine as chromogen. The sections were counterstained with Mayer's hematoxylin. Negative controls were obtained by omitting the primary antibody. As positive control, samples of carcinoma of the colon known to be p53 positive were used.

Flow Cytometry

DNA ploidy was determined according to the method of Hedley et al. [25]. Histograms with a full peak coefficient of variation >8.0% for the G0/G1 population were discarded. Cell cycle analysis was performed using the Multicycle Software Program (Phoenix Flow Systems, San Diego, CA) based upon the polynomial S-phase algorithm [26], with an iterative, nonlinear, least-squares fit [27]. Tumor proliferative activity was expressed in terms of the calculated percentage of cells in the S-phase of their cycle.

Statistical Analysis

Patient and tumor characteristics were compared between p53 positive and negative groups with Chi-square and Student's *t*-tests. The parameters that showed a significant association ($P < 0.05$) with p53 positivity were entered in a logistic regression analysis to identify the subset of variables that might discriminate the two groups.

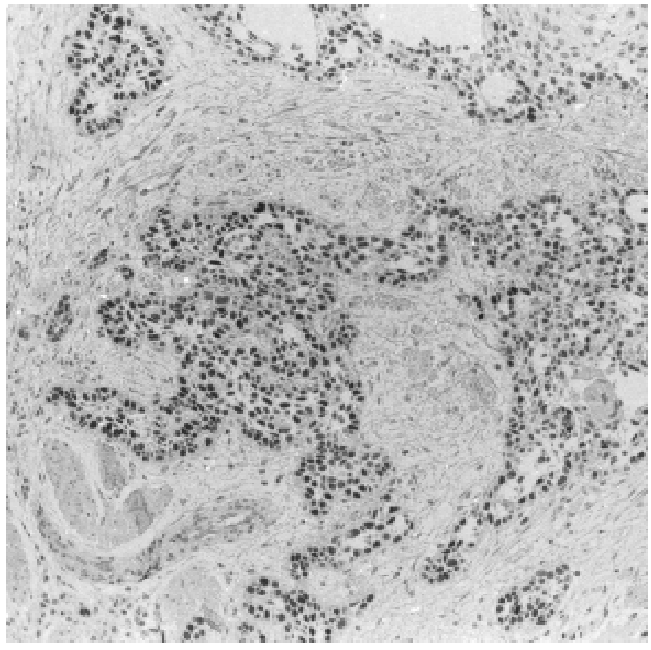

Fig. 1. Squamous cell carcinoma infiltrating the muscular layer; >90% of the nuclei of the neoplastic cells express p53 protein.

RESULTS

In 21 of 37 cases (56.8%), there was strong brown nuclear staining of the neoplastic cells assumed to be specific for p53 protein accumulation (Fig. 1). The remaining 16 cases (43.2%) were considered to be negative. This group included cases with few scattered, weakly stained, neoplastic cells but amounting to <10% of the total number of the neoplastic elements (Fig. 2).

The clinical characteristics of the two groups of patients, with p53-positive and p53-negative neoplasias, respectively, are summarized in Table I. No difference in prevalence was found between the two groups in relation to sex, mean age, location of the tumor, presence of stenosis, alcohol consumption, and pre-operative radiotherapy. A positive correlation between p53 immunoexpression and tobacco consumption was found ($P = 0.02$).

The histopathological and cytometric characteristics of the two groups of tumors are mentioned in Table II. Tumor invasion beyond the muscular propria (T3 + T4 cases) was found in 20 of 21 p53 positive tumors (95.2%) and in 9 of 16 tumors of the negative group (56.3%) ($P = 0.01$). Perineural tumor growth was present in 13 of the 21 positive tumors (61.9%) in contrast to the p53 negative group in which it was demonstrated only in four tumors (25.0%) ($P = 0.04$). Both lymphatic permeation and venous invasion were more common in the group of tumors with p53 positivity (18 vs. 9) and (3 vs. 1), respectively, but the differences have no statistical significance. The two groups of tumors do not show statistically

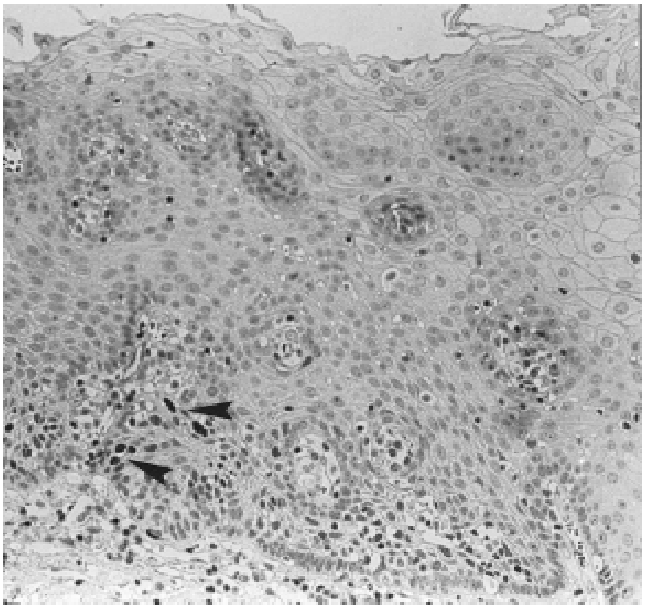

Fig. 2. Normal esophageal mucosa adjacent to carcinoma. Very few nuclei are stained for p53 (arrow).

TABLE II. Pathological and Cytometric Characteristics of p53 Positive and Negative Groups of Patients With Esophageal Squamous Cell Carcinomas

	p53 positive (%)	p53 negative (%)	*P*
Tumor (n)	21(56.8)	16(43.2)	
Grade (G)			NS
G1	16(76.2)	11(68.8)	
G2	4(19.0)	3(18.8)	
G3	1(4.8)	2(12.4)	
Local invasion (T)			0.01
T1	0(0.0)	1(6.2)	
T2	1(4.8)	6(37.5)	
T3	19(90.5)	8(50.0)	
T4	1(4.7)	1(6.3)	
Lymphatic permeation			NS
yes	18(85.7)	9(56.2)	
no	3(14.3)	7(43.8)	
Venous invasion			NS
yes	3(14.3)	1(6.3)	
no	18(85.7)	15(93.7)	
Perineural growth			0.04
yes	13(61.9)	4(25.0)	
no	8(38.1)	12(75.0)	
DNA content			NS
Diploid	6(28.6)	8(50.0)	
Aneuploid	15(71.4)	8(50.0)	
S-phase (mean)	26.5 ± 10.3	24.6 ± 11.5	NS

significant differences regarding the other parameters evaluated (Table II), i.e., grading, ploidy, and S-phase fraction. Multivariate analysis with logistic regression (Table III) showed that tumor invasion was the only factor that discriminated positive from negative p53 cases. Tumor adjacent mucosa was diploid in all the cases

TABLE III. Multivariate Analysis With Logistic Regression

	Odds ratio	*P*	95% confidence intervals
Local invasion (T)[a]	9.3	0.08	0.0–119.0
Lymphatic permeation	0.3	0.23	0.0–2.1
Venous permeation	1.0	0.94	0.0–14.8
Perineural growth	0.5	0.47	0.0–3.0
Local invasion (T)[b]	15.6	0.02	1.5–158.0

[a]The upper part of the table shows the results for the parameters with statistical significance.
[b]The lower part of the table shows the result when only local invasion (T) was considered.

whether it had normal features, koilocytic or dysplastic changes.

Normal mucosa stained negatively for p53, except for a few scattered basal cells that presented weak nuclear staining. A similar finding was demonstrated in nondysplastic mucosa exhibiting koilocytic features (9 of 12 cases), where the scattered p53 positive epithelial cells were also found in the basal cell layer (Fig. 2), differently from the squamous cells that exhibited perinuclear halo and eosinophilic cytoplasmic changes beneath the superficial layer. Sixteen mucosal areas where dysplastic changes were identified contained p53 immunostaining in one low-grade area and six high-grade areas (Fig. 3). In one case, positive low-grade and high-grade dysplasia were observed (Fig. 4). The p53 positive dysplastic epithelium was always observed in continuity with p53 positive carcinoma areas.

DISCUSSION

The present study demonstrates that p53 product is expressed by the squamous neoplastic epithelium of approximately half of the esophageal cancers. This frequency for p53 involvement in the esophageal carcinogenesis is most probably understimated, since it is well known that there are p53 mutations associated with nonstable proteins that remain undetected if only immunohistochemical evaluation is used [2]. The molecular mechanisms implicated in the malignant transformation of the esophageal mucosa and cancer progression remain to be identified.

Our results illustrate that p53 dysfunction is involved in the tumorigenesis of the esophagus, and this assumption is reinforced by the demonstration of p53 immunoexpression in dysplastic mucosa adjacent to neoplasia, evaluated in 7 out 16 cases. This observation confirms recent reports from Volant et al. [19] and Parenti et al. [20].

Our results also demonstrate a positive correlation between cigarette smoking and esophageal cancer associated with p53 alterations. This conclusion is in agreement with that of others [8], but contradicts Flejou et al. [28],

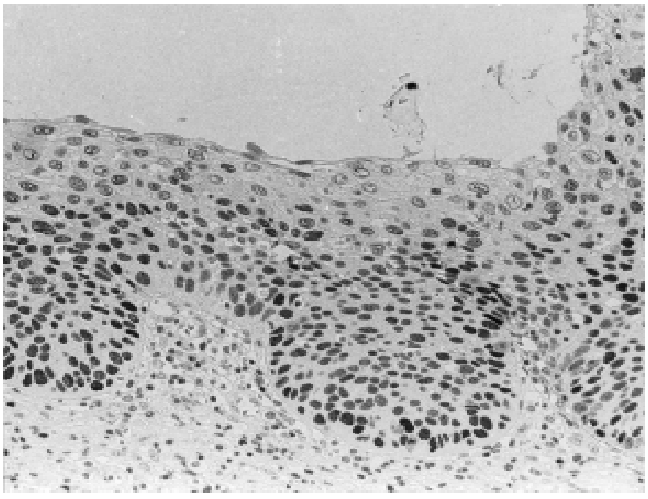

Fig. 3. Dysplastic mucosa adjacent to carcinoma; >10% of the epithelial cell nuclei exhibit p53 protein accumulation.

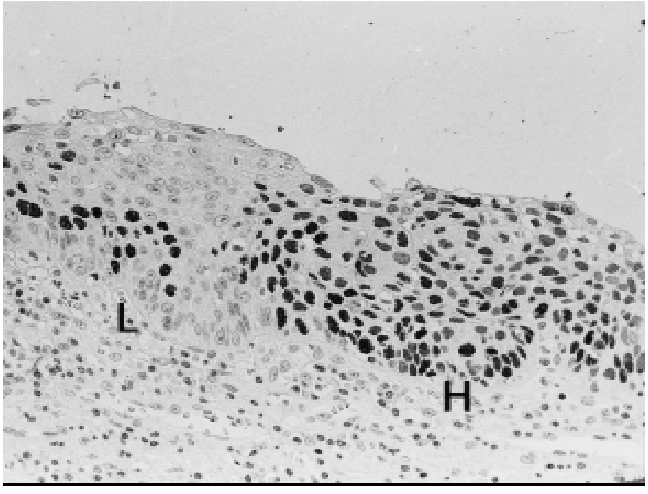

Fig. 4. Esophageal mucosa with dysplastic features. Foci of low-grade dysplastic changes (L) and high-grade dysplastic areas (H). High grade dysplasia (H) contains more positive nuclei than low-grade dysplasia (L).

who found no relationship between p53 incidence and tobacco consumption. It is well known that a wide spectrum of factors may be implicated in p53 wild-type protein inactivation and p53 gene mutation [3–5]. Cigarette smoking is one of these factors known to induce alterations in the p53 system and to originate abnormal protein products that can be detected by immunocytochemistry [8,29–34]. Attempts have been made searching for the potential prognostic value of p53 dysfunction in human cancers [9–16,35–40]. Several studies evidenced an association between p53 abnormalities, tumor progression and poor disease outcome in various neoplasia, namely, breast, prostate, brain, liver, lymphoid and head and neck [9–14,35,36]. In contrast, other studies did not demonstrate such prognostic value for p53 dysfunction in hypopharynx, esophageal, gastric, and colorectal cancers [15,16,37–40].

By univariate analysis, p53 overexpression correlated positively with tumor invasion and perineural neoplastic spread (Table III). Lymphatic permeation was also more common in the p53 positive group, but this association ($P = 0.06$) did not reach statistical significance (Table III).

By multivariate analysis, local tumor invasion proved to be the single parameter that discriminated between negative and positive p53 groups of tumors (Table III). It is known that neoplastic infiltration of the esophageal wall is a major prognostic factor in esophageal squamous cell carcinoma. Therefore, the statistically significant correlation of p53 immunoexpression and tumor invasion points to associate p53 changes with local progression and potential aggressiveness. Likewise, it is tempting to suggest that the immunocytochemical p53 assessment in biopsy samples might be used as a tumor biomarker to help in the planning of the treatment of patients.

In contrast to our results, Sasano et al. [39] found no correlation between p53 immunoexpression and the clinicopathologic parameters commonly associated with the biologic behavior of squamous cell esophageal carcinoma, namely, grade, clinical stage, tumor size, depth of invasion and vascular permeation. Sarbia et al. [16] also did not find p53 expression to be correlated with T, nodal, and grading status. We also did not find association of p53 immunoexpression and tumor grade, and the same was verified for DNA nuclear ploidy and S-phase fraction. The percentage of diploid tumors was much lower in the p53 positive group in comparison with the p53 negative group (29% vs. 50%), but the difference had no statistical significance. This can be due to characteristics of the samples used for cytometric assessment and also can be influenced by the technique itself, which does not discriminate accurately between diploid and near-diploid neoplasms.

Normal esophageal mucosa adjacent to tumor was consistently negative for p53 with the exception of a few scattered cells in the basal epithelial layer found to have positive nuclear staining. Those scattered cells were not sufficient in number for classifying the cases as positive, since our quantitative criteria used the cut-off value of 10% stained nuclei. The adjacent mucosa of nine cases that displayed features compatible with a cytopathic viral effect also showed a few stained nuclei in the basal layer of the epithelium. Scattered stained nuclei were also described in HPV infected mucosa of the uterine cervix, and this finding was explained by the prolonged wild-type protein half-life due to increased proliferation rate of the epithelial basal cell compartment [21]. It is well known that immunohistochemistry is not an accurate method for the evaluation of the frequency of p53 dysfunction associated with HPV infection since p53 nega-

tive results do not exclude the presence of molecular alterations induced by the virus. Controversy still exists on the effect of complex formation between E6 viral domain and p53 whether promoting the rapid proteolytic degradation of p53 protein through ubiquitin pathway [41] or leading to its longer half-life [42].

The p53 positive dysplastic areas found in seven cases were detected exclusively in the group of tumors that also exhibited p53 immunostaining. The higher number of positive high-grade dysplastic areas in relation to low-grade areas (6 vs. 1) may be associated with progressive accumulation of abnormal p53 gene product confirming previous observations by Wang et al. [20]. Similar to Volant et al. [19], we also found in dysplastic areas a progressive distribution pattern of the p53 positive cells from the basal cell layer to the upper cell layers. This observation excludes the interpretation of colonization of adjacent mucosa by p53 positive carcinoma cells and further suggests that basal cells may be the earliest site of disturbance in esophageal carcinogenesis. Assuming that esophageal carcinogenesis is a multistep process, these observations favor that p53 changes are implicated in the preinvasive stages and, therefore, play a role in the initiation and/or the early phases of cancer progression.

An identical progression in p53 immunodetection has been observed in other premalignant lesions related to squamous cell carcinomas, which suggest that p53 mutation may be an early genetic event involved on the malignant development of this type of epithelium [19,20,43]. This contrasts with the association of p53 alterations and advanced tumor stages demonstrated in neoplasias of glandular and transitional origin [44–48].

However, contrary to other types of carcinomas with squamous histogenesis, we also found p53 positivity significantly associated with advanced stages of esophageal cancer, allowing us to hypothesize that p53 identifies a clonal evolution with a more aggressive phenotype expressed even in the earliest of the carcinogenic process.

In conclusion, our study indicates that p53 dysfunction is involved in early stages of the malignant transformation of the esophageal epithelium but also seems to be associated with further progression of a subset of the neoplasia with a greater invasive capacity.

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